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NS_2 protein of influenza virus is found in purified virus and phosphorylated in infected cells

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Summary. Purified viral preparations of influenza A virus were examined for the presence of NS_2 protein hitherto considered as a viral nonstructural protein that is present only in infected cells. Analysis of purified virus by radioimmunoprecipitation with monospecific antisera to NS_2 revealed its presence in the virus particle suggesting that it is a viral structural protein. NS_2 protein was also shown to be phosphorylated in infected cells in this study. This brings the number of influenza virus phosphoproteins to three which include NP, NS_1, and NS_2. These observations raise important questions about the role of NS_2 in the replication of influenza virus.

Introduction

The genome of influenza A virus (familiy *Orthomyxoviridae*, type A) consists of eight negative sense single stranded RNA segments which encode ten known viral specific polypeptides [12]. The smallest segment, segment 8, codes for two proteins NS_1 and NS_2 which are considered to be nonstructural proteins [7,10–12]. The NS_1 protein is translated from a colinear transcript of the RNA segment 8, whereas the NS_2 protein is synthesized using a spliced mRNA derived from NS_1 mRNA. Both NS_1 and NS_2 share the same nine amino acid amino-terminal coding region but differ in the rest of their amino acid sequence because different reading frames are used after the splice junction [12]. NS_1 is made in abundant amounts early in infection whereas NS_2 is synthesized in small quantity during the late phase [9, 13]. Both proteins migrate into the infected cell nucleus where virus replication is known to occur [6, 17, 18]. NS_1 protein was shown to be phosphorylated in infected cells although the role of phosphorylation in its function remains to be determined [14]. Although much is learned about

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the structures and sequences of the nonstructural proteins [7, 10–13], very little data exists on their role in viral multiplication. The only available evidence for their participation in viral replication was obtained from the studies on temperature-sensitive (ts) mutants of influenza virus that are defective in the NS gene segment [8, 15, 17]. In cells infected with these ts mutants, virus multiplication does not proceed beyond primary transcription and translation indicating that NS gene products play a role during the steps of vRNA synthesis.

Although NS_2 is referred to as a nonstructural protein that is present only in infected cells [12], during our previous work with defective influenza virus coded proteins [1, 2] we consistently observed a protein of the expected molecular weight of NS_2 ($\sim$ 12 kDa) in purified viral preparations (R. K. Akkina, unpubl. obs.). In addition, early work by Lamb et al. showed that NS_2 could be detected in purfied virus although this finding was overlooked during the subsequent years [13]. Similarly, the M_2 protein of influenza virus which was previously considered to be a nonstructural protein, has recently been shown to be indeed present in purified virus albeit in low amounts [19]. Because of these observations, we re-examined the possibilty that NS_2 could be a viral structural protein using the recently developed monospecific antisera. Since phosphorylation is an important post-translational modification of many proteins that are involved in regulation, we therefore also examined NS_2 to see if it is phosphorylated in infected cells. In this paper, we show that NS_2 is present in purified influenza virus and that it is phosphorylated and discuss the possible implications of these findings.

Material and methods

Cells and viruses

Madin Darby bovine kidney (MDBK) cells were grown and maintained in minimal essential medium (MEM) supplemented with 5% newborn bovine serum [2]. Chick embryo fibroblasts (CEF) were prepared from 11-day old embryos as described [5] and grown in MEM supplemented with 5% bovine serum and 2% chicken serum. Influenza virus, A/WSN/33 (H1N1) was propagated in MDBK cells while the other influenza virus subtypes, A/equine/Miami/63 (H3N8), A/swine/Wisconsin/7/83 (H1N1), A/Texas/1/77 (H3N2), A/Singapore/1/57 (H2N2), and A/duck/England/56 (H11N6) obtained from Dr. Virginia Hinshaw, University of Wisconsin, were grown in 10 day old embryonated eggs for preparation of virus stocks [5].

Antisera

Monospecific antisera of rabbit origin to the influenza A virus NS_1 and NS_2 proteins used in this study were a generous gift of Dr. Peter Palese (The Mount Sinai School of Medicine, New York). These antibodies were raised against bacterially expressed NS_1 and NS_2 proteins and their specificities were previously described [6, 18]. Normal rabbit serum was used as negative control serum in immunoprecipitations.

Virus infection and radiolabeling of viral polypeptides

Confluent MDBK or CEF cell monolayers were infected with virus at a multiplicity of infection (m.o.i.) of 3–5 plaque forming units (PFUs) per cell. Viral polypeptides in infected

cells were radiolabeled with 50 μCi/ml [^{35}S]methionine for 2 h at 5.5 h postinfection (p.i.) as described previously [2]. To label phosphoproteins, infected cells were labeled with [^{32}P]H_3PO_4 (200 μCi/ml), (ICN) for 4 h at 4 h p.i. At the end of the labeling period, the cells were washed in cold PBS and processed for immunoprecipitation as described below.

Growth and purification of radiolabeled virus

MDBK or CEF cells were infected with various influenza viruses as described above and the infected cells were incubated in medium containing [^{35}S]methionine (25 μCi/ml) for 24 to 48 h, or until most of the cells were destroyed by virus infection. The radiolabeled virus in the culture supernatant was purified as described below [5]. The clarified virus containing cell culture supernatant was layered onto a 20% sucrose cushion in NTE (100 mM NaCl; 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and the virus was pelleted by centrifugation in a Beckman SW 27 rotor at 75,000 × **g** for 3 h. The virus in the pellet was resuspended in NTE and was subsequently purified by centrifugation through a linear 20%–65% sucrose density gradient for 16 h at 150,000 × **g** in a Beckman SW 41 rotor. The visible viral band from the grandient was collected, diluted in NTE and virus was pelleted in a SW 50.1 rotor at 40,000 rpm for 3 h. The viral pellet was resuspended and again purified by centrifugation through a linear gradient of 15%–60% potasium tartrate (in NTE) for 16 h at 150,000 × **g** in a SW 41 rotor. The viral band was collected, diluted in NTE and repelleted as above. The purified virus was frozen and stored at −20 °C until use.

Immunoprecipitation of viral polypeptides and electrophoretic analysis

All the immunoprecipitation steps were carried out at 0–4 °C as previously described [3]. The infected radiolabeled cells or purified radiolabeled virus were lysed in 1 ml cold lysis buffer (10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 2mM EDTA, 1% NP 40, 0.5% Na-deoxycholate, 1% aprotinin (Sigma), and 0.5 mg/l leupeptin (Sigma). The lysates were clarified in an Eppendorf centrifuge at 14,000 rpm for 30 min. SDS was added to a concentration of 0.5% and the lysates were boiled for 3 min. The lysates were subsequently cooled on ice and 5μl of antiserum was added to each 1 ml lysate and incubated overnight.

Subsequently, 10 mg protein A sepharose (Pharmacia), preswollen in 100 μl of lysis buffer, was added to the antibody-cell extract mixture and incubated for 30 min with shaking. The antigen-antibody-protein A complexes were pelleted by centrifugation and washed two times sequentially in the following buffers: (1) 1 M buffer (10 mM Tris-HCl pH 7.2, 1 M NaCl, and 0.1% NP 40), (2) RIPA buffer (10 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Na deoxycholate, 1% Triton X100 and 0.1% SDS), (3) STE (10 mM Tris-HCl pH 7.2, 150 mM NaCl, and 1 mM EDTA). Immunoprecipitates were boiled in 100 μl of SDS-PAGE sample buffer for 1 min, centrifuged and the supernatant was collected for subsequent electrophoretic analysis by SDS-PAGE in 13% gels with 4 M urea [9]. Viral proteins were detected by autoradiography.

Results

Detection of NS_2 protein in purified virus

A highly purified radiolabeled virus obtained by two consecutive density gradient centrifugations in sucrose and potasium tartrate was used for analysis of viral structural proteins. To determine if NS_2 was present in the purified virus, infected cell lysates containing NS_2, immunoprecipitates of infected cell lysates with NS_2 monspecific antiserum, and purified virus were compared in 13% SDS-PAGE gels. Results showed the presence of a 12 kDa polypeptide, presumably NS_2, in purified virus (Fig. 1, lane 4). This polypeptide band comigrated

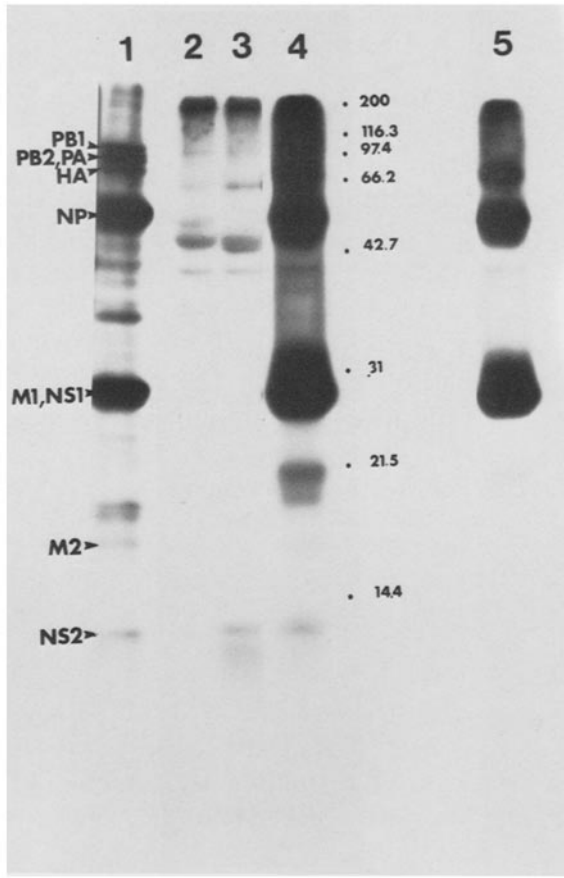

Fig. 1. Detection of NS_2 protein in purified influenza virus. [^{35}S]methionine-labeled A/WSN virus was purified as described in Materials and methods and the viral polypeptides were analysed by 13% SDS-PAGE. *1* Infected cell lysate; *2* and *3* infected cell lysates immunoprecipitated with NS_2 negative and positive antisera respectively; *4* purified virus; *5* shorter exposure of *4*. Positions of viral proteins are marked on the left and that of standard molecular weight markers (in kDa) next to *4*

with the NS_2 from infected cell lysates (Fig. 1, lane 1) as well as with the NS_2 polypeptide immunoprecipitated with NS_2 monospecific antiserum (Fig. 1, lane 3). The NS_2 migrated as a broad heterogenous band as previously described [13]. This could be due to partial degradation of the protein during experimental procedures. All of the previously determined viral structural proteins including M_2 were detectable in the purified virus although most were obscured in this exposure of the film. In addition, a broad protein band ($\sim$21 kDa) of unknown identity was seen in purified virus (Fig. 1, lane 4). This protein band was also seen in gels of highly purified influenza virus presented by others [19]. Long exposure of most of the gels had become neccesary to show the presence of NS_2 protein which was made in small quantities in infected cells. This resulted in the over-exposure of film for other viral proteins which were made in much higher amounts.

To show that the protein in purified virus that migrated at the position of NS_2 was indeed NS_2, radiolabeled purified virus was immunoprecipitated with NS_2 antiserum. Results showed that this protein is indeed present in the virus particle (Fig. 2, lane 2). The NS_2 from the purified virus comigrated with the authentic NS_2-immunoprecipitated from infected cells (Fig. 2, lane 5). To authenticate the purity of virus, immunoprecipitations were also carried out with NS_1 antisera to determine if this abudantly made nonstructural protein (levels of NS_1 protein are at least ten-fold higher than NS_2 in infected cells) copurified with virus particles as a contaminant. NS_1 protein was absent in the virus particle as shown by immunoprecipitation of purified virus with NS_1 antiserum (Fig. 2, lane 1). The absence of NS_1 in purified virus served as an indicator of purity of the virus preparation. A small amount of M_1 protein was seen in the

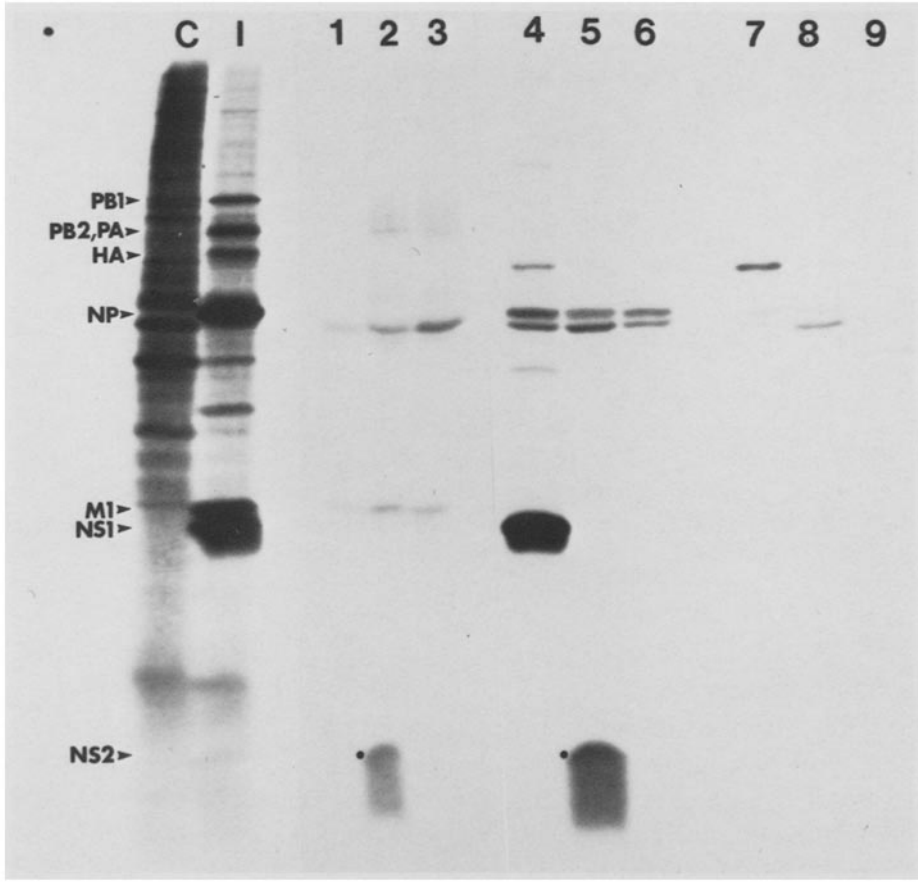

Fig. 2. Immunoprecipitation of NS_2 protein from purified virus. [^{35}S]methionine-labeled purified A/WSN virus was immunoprecipitated with NS_1 and NS_2 monospecific antisera as described in text and analysed by 13% SDS-PAGE. *C* and *I* Uninfected and infected cell lysates: *1–3* purified virus immunoprecipitated with NS_1, NS_2, and negative control sera, respectively; *4–6* infected cells immunoprecipitated with NS_1, NS_2, and negative control sera respectively; *7–9* uninfected cells immunoprecipitated with NS_1, NS_2, and negative control sera respectively. ● Positions of NS_2

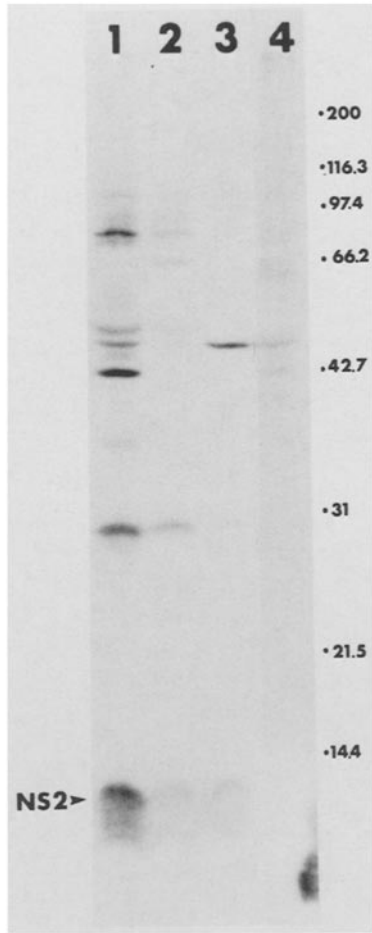

Fig. 3. Immunprecipitation of NS_2 protein from purified virus preparations of influenza viral subtypes. [^{35}S]methionine-labeled purified virus preparations of different influenza viral subtypes were immunoprecipitated with NS_2 antisera and analysed by 13% SDS-PAGE. *1* A/Texas/1/77; *2* A/Singapore/1/57; *3* A/duck/England/56; *4* A/Texas/1/77 virus immunoprecipitated with control negative serum

immunoprecipitations of purifed virus as a nonspecific contaminant (Fig. 2, lanes 1–3). We also considered the possibility that presence of NS_2 in purified virus could be an artifact of the cell culture derived virus. Therefore, virus grown in allantoic cavity of 10 day old embryonated chicken eggs was examined by Western blotting technique for the presence of NS_2. Results showed that NS_2 was indeed present in these preparations as well (data not shown) indicating that packaging of NS_2 protein into virus particles is a selective event in virus assembly.

To determine if packaging of NS_2 protein into virus particles is conserved among influenza viral subtypes, three other influenza viruses were examined by immunoprecipitation with NS_2 antisera. Results showed that NS_2 is also

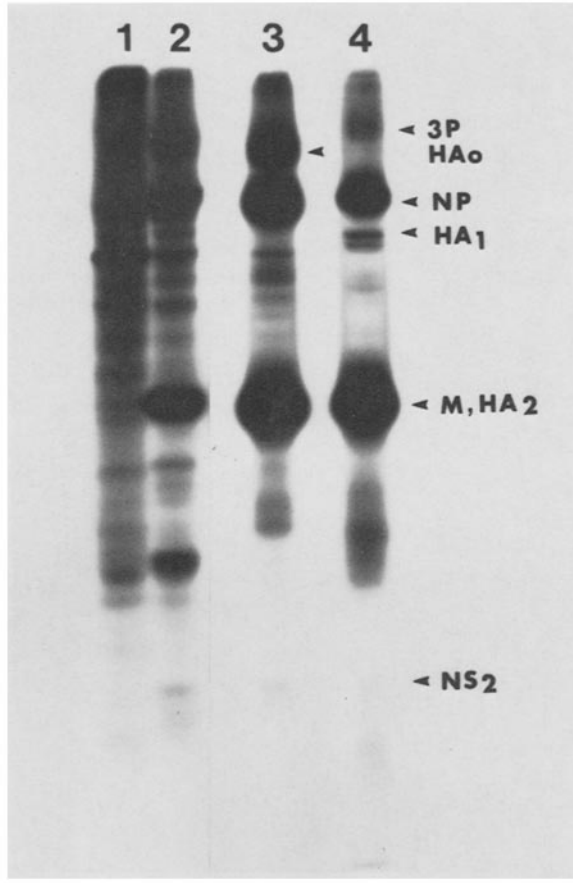

Fig. 4. Detection of NS_2 protein in purified virus subjected to trypsin digestion. A/WSN virus was radiolabeled with [^{35}S]methionine and purified by centrifugation as described in text. The purified virus was then subjected to digestion with TPCK trypsin (20 μg/ml) in NTE for 30 min at 37 °C. The digested virus was pelleted by centrifugation and analysed by 13% SDS-PAGE. *1* and *2* Uninfected and infected cell lysates: *3* purified virus; *4* purified virus digested with trypsin. The three polymerase proteins are designated as 3P in *4* and uncleaved HA as HAo in *3*

present in purified A/Texas/1/77 (H3N2), A/Singapore/1/57 (H2N2), and A/duck/England/56 (H11N6) influenza viruses (Fig. 3, lanes 1–3).

Since these results suggested that NS_2 is a viral structural protein, it was of importance to determine its location in the virion. NS_2 was previously shown to be trypsin sensitive [13]. Therefore, the purified virus was subjected to trypsin digestion with the assumption that if NS_2 is present on the virus surface, it should be susceptible to the enzyme treatment. Analysis of purified virus by SDS-PAGE after trypsin digestion showed that it remained intact indicating that it is probably located within the virus particle (Fig. 4, lane 4). In contrast, the viral surface glycoprotein HA was completely cleaved to HA_1 and HA_2 subunits as expected (Fig. 4, lanes 3 and 4).

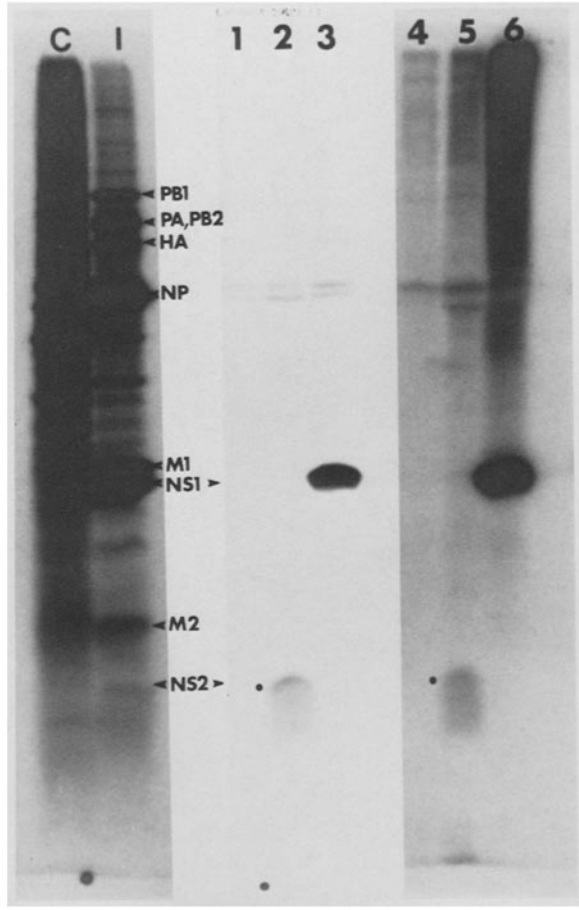

Fig. 5. Phosphorylation of NS_2 protein in infected cells. A/WSN virus infected cells were labeled with ^{32}P-orthophosphoric acid or [^{35}S]methionine for 4 h at 4 h p.i. and immunoprecipitated with NS_1 and NS_2 monospecific antisera. The immunoprecipitates were analysed by 13% SDS-PAGE. *C* and *I* [^{35}S]methionine-labeled uninfected and infected cell lysates: *1–3* [^{35}S]methionine-labeled infected cells immunoprecipitated with negative control serum, NS_2 and NS_1 monospecific antisera respectively; *4–6* ^{32}P-orthophosphoric acid labeled infected cells immunoprecipitated with negative control serum, NS_2, and NS_1 antisera respectively. ● Positions of NS_2

Phosphorylation of NS_2 protein

To determine if NS_2 is phosphorylated in infected cells, cells were labeled with ^{32}P-orthophosphoric acid and immunoprecipitated with the NS_2 monospecific antisera as described in the Materials and methods. Results showed that NS_2 incorporated the ^{32}P label indicating that it is a phosphoprotein (Fig. 5, lane 5). Since NS_1 was previously shown to be phosphorylated [14], it was used as a positive control for phosphorylation (Fig. 5, lane 6). The ^{32}P incorporated NS_2 protein migrated as a broad heterogenous band similar to the one labeled with ^{35}S methionine (Fig. 5, lanes 2 and 5). To determine if phosphorylation

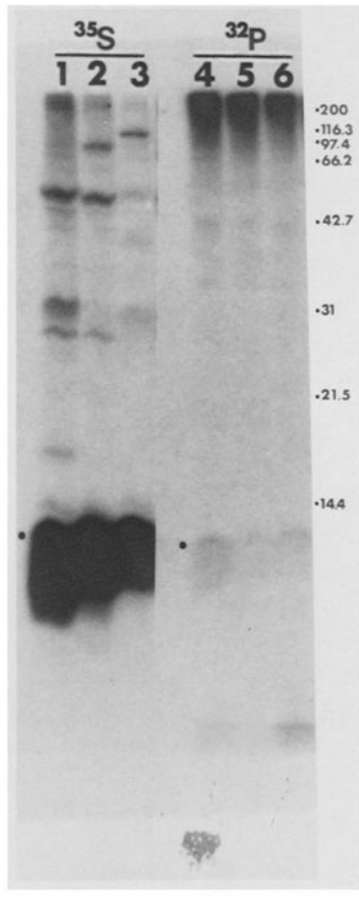

Fig. 6. Phosphorylation of NS_2 protein in various influenza viral subtypes. Cells infected with different influenza viral subtypes were labeled with [^{35}S]-methionine (*1–3*) or ^{32}P-orthophosphoric acid (*4–6*) as described in text, immunoprecipitated with NS_2 antiserum and analysed by 13% SDS-PAGE. *1* and *4* A/WSN virus; *2* and *5* A/equine virus; *3* and *6* A/swine virus

of NS_2 is conserved among other influenza viral subtypes, two type A animal influenza viruses, A/equine and A/swine, were also examined. Our results showed that NS_2 proteins of these two viruses also are phosphorylated (Fig. 6, lanes 5 and 6). It was necessary to expose the gels for a relatively long duration (2–3 weeks) to demonstrate phosphorylation of NS_2 protein. As can be seen, the NS_2 bands appear overexposed in lanes labeled with [^{35}S]methionine (Fig. 6, lanes 1, 2, and 3) compared to the ones labeled with ^{32}P-orthophosphoric acid (Fig. 6, lanes 4, 5, and 6). This suggests that only a certain population of NS_2 protein is phosphorylated in infected cells.

Discussion

Current literature on influenza virus always refers to NS_2 as a nonstructural protein that is found only in the infected cells [12]. In the present study using

monospecific antisera we showed that NS_2 is present in the purified virus preparations indicating that it is a viral structural protein. The packaging of NS_2 into virus particles is conserved among influenza viral subtypes as demonstrated by its presence in all of the three different viral subtypes, i.e., A/Singapore (H2N2 virus), A/Texas (H3N2 virus), and A/duck/England (H11N6 virus) viruses that were tested, in addition to the A/WSN virus. This confirms the earlier observations of Lamb et al. [13] with A/WSN influenza virus. The recent availability of NS_2 specific antiserum [6] permitted a more sensitive and thorough analysis of purified virus which was not possible in earlier studies. We also showed that NS_2 is most likely an internal protein of the virus particle, since it is not susceptible to trypsin digestion of the whole virus. Laser densitometry scanning of autoradiographs of the purified virus and comparison of intensity of the NS_2 band to that of HA_1 after normalizing the values according to their known methionine content, allowed preliminary quantification of the NS_2 protein in the virus particle. Our rough estimates obtained by this method indicated that about 120 molecules are present in the virion. These observations lead to the obvious questions about the role of the NS_2 protein in the virus particle and in viral multiplication steps. Previous studies showed that NS_2 is made during late phases of virus infection [13]. For the synthesis of NS_2 to occur, prior translation of viral proteins from early transcripts derived by primary transcription of the input viral genomic segments is necessary. Therefore, the functions of NS_2 protein during primary transcription, and/or early replication steps, if any, would not be available for the input virus unless it is present in the virus particle. Future studies that involve in vitro transcription/replication experiments with purified virus or infected cell extracts, and inhibition of these reactions by NS_2 antisera should yield more information on the role of NS_2, if any, in these synthetic steps.

We also showed that NS_2 is phosphorylated in infected cells and that this property is conserved among influenza viral subtypes. We have not determined if phosphorylated NS_2 is packaged into mature virus particles. Previous attempts to detect phosphorylation of NS_2 were unsuccessful primarily because these studies were done using total infected cell extracts which have a high degree of host cell background [13]. In the present study, use of NS_2 monospecific antiserum permitted specific isolation of NS_2 by immunoprecipitation. So far the only other influenza viral proteins that were shown to be phosphorylated are NS_1 and NP [12, 14]. The present finding brings the number of influenza viral phosphoproteins to three. Functional studies involving the NS_2 in in-vitro replication systems would have to be carried out in the future to determine the precise role of NS_2 phosphorlylation in viral replication. In addition, it would be interesting to determine if some of the temperature sensitive mutants with lesions in the NS gene segment [8, 17] are deficient in NS_2 phosphorylation and what effect this would have on viral replication. In vesicular stomatitis virus (VSV), another negative sense RNA virus, phosphorylation of the NS protein has a direct role in virus replication [4] and an analogous process may

conceivably operate in influenza virus. Clearly, further studies on NS_2 must be carried out before any meaningful conclusions about the significance of its incorporation into the virus particle, phosphorylation, and possible function(s) can be drawn.

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